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TITLE: Novel Transgenic Mouse Model for Testing the Effect of Circulating IGF-I on Mammary Stem/Progenitor Cell Number and Tumorigenesis

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14. ABSTRACT
Epidemiological evidence indicates that high levels of circulating IGF-I (within the normal range) predict risk of breast cancer. To examine this experimentally, investigators have previously injected mice with IGF-I and shown that this increases mammary cancer incidence and progression. However, injection of IGF-I may create non-physiological peaks of IGF-I in the circulation. In this proposal we tested whether transgenic mice (TTR-IGF-I) that had a 30% increase in circulating levels of IGF-I (via liver specific expression) had altered ErbB2-induced mammary tumorigenesis. We found no difference in time to tumor formation in ErbB2 vs. TTR-IGF-I/ErbB2 transgenic mice. Our conclusion is either that ErbB2-induced tumorigenesis is insensitive to circulating IGF-I, or other studies using injected IGF-I were confounded by the transient spike in IGF-I caused by the non-physiologic method of administration. Further studies using additional mouse models are required to definitively address a role for circulating IGF-I in mammary tumorigenesis, however, our data suggest that the IGF-I may not directly regulate risk, but rather may be an indicator of another risk factor.

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A) INTRODUCTION

Epidemiological evidence indicates that women with IGF-I levels at the higher end of the normal range have increased risk of breast cancer[1]. A better understanding of IGFs endocrine action in breast cancer may lead to the development of new and better therapies to reduce breast cancer incidence and increase survival by lowering serum IGF-I levels.

A recent publication in Science, has shown that the loss of imprinting of IGF-II (which causes an increase in circulating IGF-II and causes increased body growth) alters intestinal maturation and tumorigenesis by increasing the stem/progenitor cell population[2]. Given that IGF-I is clearly involved in cancer progression (via its regulation of apoptosis and proliferation) this new evidence indicating a possible role in stem/progenitor cells, puts IGFs at a critical intersection of many aspects of tumorigenesis. Clearly, studies on the effect of IGF-I on stem cell survival in breast cancer are limited, at best, and require novel *in vivo* approaches to decipher any causal relationship.

We hypothesized that *circulating liver-produced endocrine IGF-I can reach and stimulate the normal mammary gland, and that this will increase stem/progenitor cell number, and increase tumorigenesis in a mouse that has a predisposing oncogene.*

In this concept award we proposed the following specific aims:

1. Analyze the effect of circulating IGF-I levels on mammary stem/progenitor cells using flow sorting and transplantation assays.
2. Examine in mouse models whether increased circulating levels of IGF-I promote mammary tumor development in MMTV-ErbB2 transgenic mice, and if this is associated with increased stem/progenitor cell numbers in premalignant lesions and tumors.

B) BODY

Aim 1) Analyze the effect of circulating IGF-I levels on mammary stem/progenitor cells using flow sorting and transplantation assays.

Before starting complex mammary stem/progenitor and transplantation assays, we first confirmed that our transgenic mice had increased circulating IGF-I and examined if this affected mammary ductal development.

Transgenic mice that overexpress the mouse IGF-I gene in the liver (TTR-IGF-I), were found to have elevated circulating IGF-I (Figure 1). Transgenic female mice (941.22 ±41.77 ng/ml) had a 27% increase in serum IGF-I levels compared to age matched wild-type littermate controls (743.69 ± 28.24ng/ml). Importantly, this increase is similar to that which confers increased risk in women.

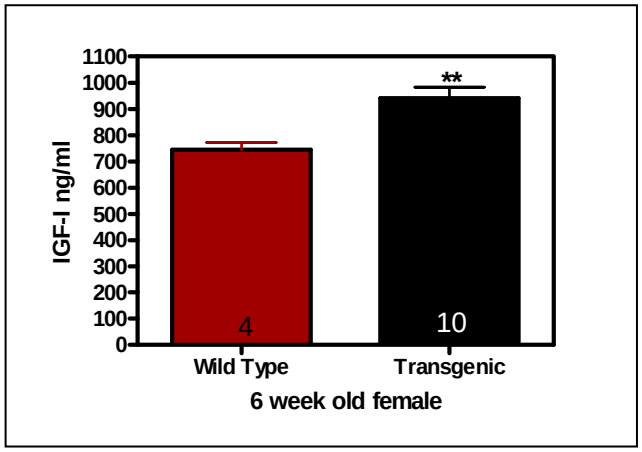

Figure 1. Increased circulating serum IGF-I levels in 6 week old female TTR-IGF-I transgenic mice. Transgenic TTR-IGF-I mice (black bar) had significantly higher ($p<0.01$) circulating levels of IGF-I compared to wild type controls (red bar). Bars indicate the mean (±SEM) serum levels of IGF-I assayed by IEMA. The number of animals is represented within the bars. **$p<0.01$

An analysis of ductal development in these animals indicated no obvious alterations by whole mount analysis (Figure 2a) or histologically (Figure 2b). We concluded that increased systemic IGF-I had no effect on ductal outgrowth, ductal side branching or ductal structure by 6 weeks of age. This is consistent with a recent publication indicating that local production of IGF-I, and not circulating IGF-I, affects mammary gland development [3].

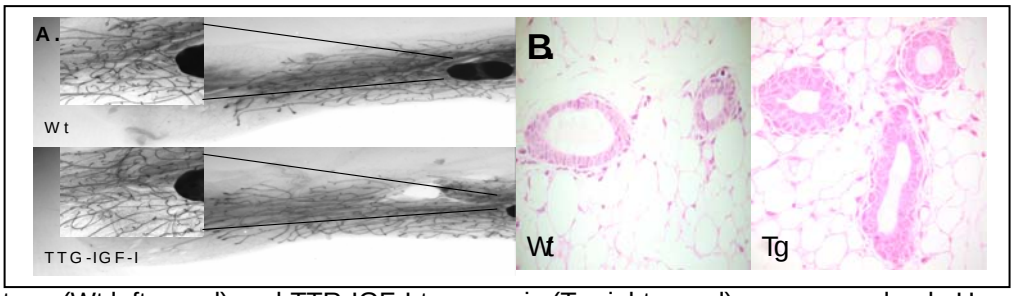

Figure 2. No effect of circulating IGF-I on mammary ductal development at 6 weeks of age. A.) Representative whole mounts of the #4 inguinal mammary gland from wt (top panel) and transgenic (bottom panel). Note no change in ductal growth or branching (insert). B.) Representative wild type (Wt;left panel) and TTR-IGF-I transgenic (Tg;right panel) mammary glands Hematoxylin and Eosin (H&E) stained by IHC. Magnification: 40x

To perform mammary gland transplantation assays to asses stem cell number and regeneration we needed to backcross the TTR-IGF-I mice into a uniform genetic background (we chose FVB/N), as these mice were on a mixed genetic background that would have caused rejection following transplantation. FVB/N was a convenient genetic strain as this is the strain for MMTV-ErbB2 mice we used to cross in Aim 2 (see next Aim). Unfortunately, backcrossing the TTR-IGF-I transgenic mice into the FVB/N background took longer than expected to complete. We are currently at F7, and generally we start transplantations when we have reached F10. This part of the Aim has thus been delayed but we expect to start the transplantations during a 1 yr no cost extension.

Aim 2) Examine in mouse models whether increased circulating levels of IGF-I promote mammary tumor development in MMTV-ErbB2 transgenic mice, and if this is associated with increased stem/progenitor cell numbers in premalignant lesions and tumors.

To study the effect of circulating levels of IGF-I on mammary tumor incidence we crossed our TTR-IGF-I (tg/wt) mice (F5 FVB/N backcross) with heterozygous MMTV-ErbB2 (tg/wt) and compared time to mammary tumor formation in the resulting offspring (Figure 3). Briefly, MMTV-ErbB2 mice are based on a mammary specific overexpression of the ErbB2 receptor, a frequently amplified oncogene in human breast cancer. The resulting offspring had one of 4 genotypes; increased systemic IGF-I only (tg/wt), mammary specific ErbB2 overexpression only (tg/wt), IGF-I and ErbB2 bigenic expression (tg/tg), or wild type controls (wt/wt).

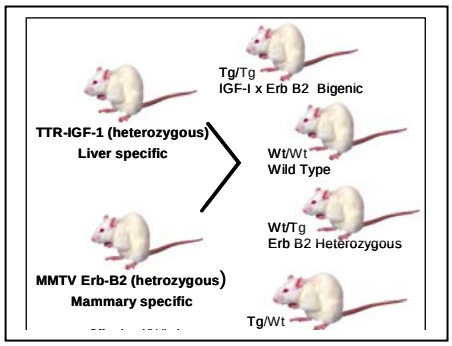

Figure 3. Model depicting the genetically different mouse lines derived from TTR-IGF-I crossed with MMTV ErbB2. Note offspring are all virgins for tumor study.

To determine the penetrance of mammary tumor formation in these groups, we measured time to tumor formation (palpable tumor >100mm^3) and using Kaplan-Meier analysis (Figure 4). When tumors reached 1cm^3 in size, animals are injected with BrdU (100mg/kg) for 2 hrs, sacrificed, and mammary glands with tumors processed for paraffin blocks or frozen in liquid nitrogen. TTR-IGF-IxErbB2 bigenic virgin transgenic female mice showed palpable mammary tumors beginning at 24 weeks of age and with a mean time to tumor formation (MMTF) of 32 weeks (Figure 4). ErbB2 only virgin transgenic mice showed a similar tumor formation with the earliest mammary tumors palpated at 24 weeks of age and a MMTF of 33 weeks of age as depicted in Figure 4. Surprisingly, circulating levels of IGF-I did not seem have an affect on tumor incidence in females overexpressing ErbB2 compared to ErbB2 only animals. Mimicking the control group (wt/wt), a palpable tumor has not yet been detected in the IGF-I only (currently the youngest female being 34wks and oldest 66 wks old).

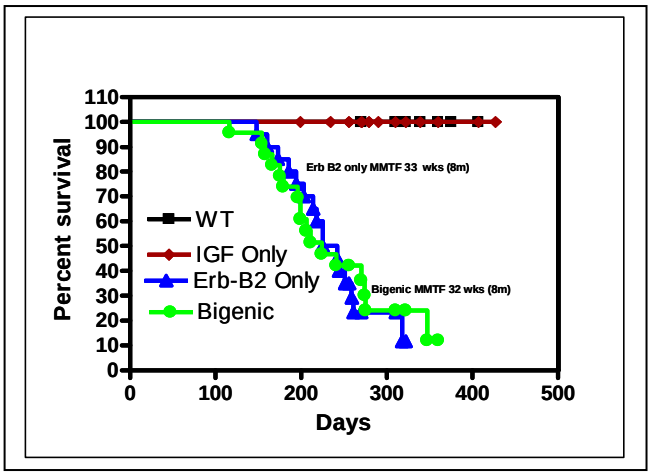

Figure 4. Elevated circulating IGF-I (TTR-IGF-I) doesn't affect mammary tumor formation in MMTV-ErbB2 virgin mice. Kaplan-Meier tumor curves that illustrate the percent of virgin bigenic ErbB2xIGF-I (green), ErbB2 only (blue), or IGF-I only (maroon) transgenic mice that are tumor free compared to wild type (WT; black) virgin controls. Note that there is no difference between the ErbB2 only and Bigenic groups, however both lines had significant increases in mammary tumor growth vs. wild type and IGF-I animals. In addition, circulating levels of IGF-I did not induce mammary tumor formation in the IGF-I only group. Steps on the graph indicate when a tumor was first palpated on each animal in the study. MMTF=mean time to tumor formation

Further analysis comparing the tumors from the ErbB2 only and bigenic group revealed that both had visible lung metastasis and approximately the same number of mammary tumors per animal (Table 1). However a preliminary analysis of the time it took a tumor to reach approximately $1cm^3$, revealed that elevated IGF-I may stimulate a faster tumor growth rate in the bigneic (7 week average) compared to the ErbB2 only group (8.7 wk avgerage). However, this is preliminary as not all of the mice have tumors, and the tumor growth rate (time to reach $1cm^3$) is confounded by different sizes at palpation and at harvesting. The tumor growth curves have are currently being analyzed by the Biostatistics core the Breast Center at BCM to see if there is a significant difference in tumor growth rate (Table 1).

Table 1. Physical comparison of tumor formation in ErbB2 only vs. bigenic (IGF-I x ErbB2) virgin females. Both group exhibit very similar tumor incidences, however IGF-I seems to induce a slightly more rapid tumor group. %= percentage of animals with tumors. Number in parenthesis indicates total number of animals per group/number out side parenthesis indicate number of animal per group with palpable tumors.

	Erb-B2 Only	IGF-1 x Erb-B2
Number of Tumors	16(20) 80%	17(23) 74%
MTTF	33 wks/ 8.25m	32 wks/ 8m
Lung Metastasis	yes	yes
Av. # of Tumors per Animal	1.9	2.00
Av. Tumor Growth Rate	8.70 wks	7.01 wks

The potential difference in growth of tumors in bigenic ErbB2xTTR-IFG-I mice compared to TTR-IGF-I mice is exciting given that mammary tumors in the bigenic virgin females had significantly ($p<0.05$) higher IGF-I protein levels compared to tumors from ErbB2 only females at the time of sacrifice (Figure 5 far right panel). Importantly, there was no difference in IGF-I protein levels in normal mammary glands (no detectable tumor) from the same animals between the two groups even though (Figure 5, middle panel), as expected, serum IGF-I levels were significantly higher ($p < 0.05$) in these bigenics compared to ErbB2 only females (Figure 5 left panel).

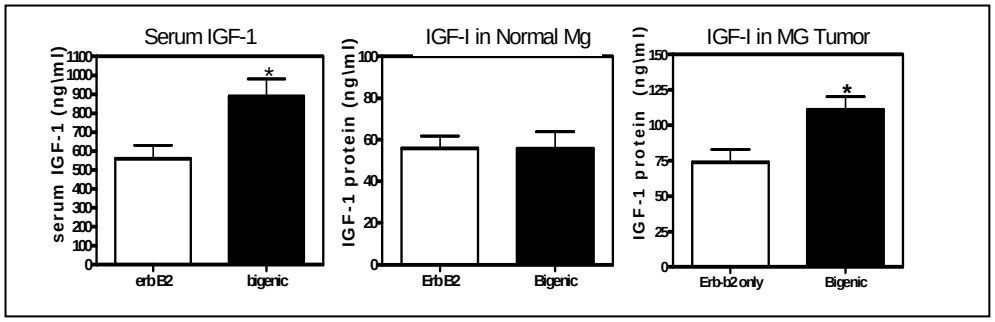

Figure 5. Comparison of IGF-I protein and serum levels in tumors and mammary glands from ErbB2 only and bigeneic virgin females. Increased circulating levels on IGF-I resulted in significantly higher ($p < 0.05$) IGF-I protein levels in tumors from bigenic females compared to their respective normal mammary glands and to normal mammary glands and tumors from ErbB2 only transgenics. Bars indicate the mean (±SEM) serum or protein levels of IGF-I assayed by IMEA. * = $p < 0.05$

SUMMARY

By crossing mice with elevated circulating IGF-I (TTR-IGF-I) with mice with a predisposing mammary specific oncogene (MMTV-ErbB2) we have provided evidence that circulating levels may not play a role in

initiating mammary gland tumorigenesis. This raises significant questions regarding epidemiological studies indicate that circulating IGF-I levels predict breast cancer risk. However preliminary evidence suggests that circulating IGF-I may enhance tumor growth rate. In a no-cost extension we will specifically test whether IGF-I is regulating mammary stem cell number using transplantation assays.

C) KEY RESEARCH ACCOMPLISHMENTS

- Elevated circulating IGF-I levels in TTR-IGF-I mice
- Increased circulating IGF-I doesn't alter mammary gland development in female virgin mice
- No change in time to tumor formation in TTR-IGF-IxMMTV-ErbB2 mice vs. MMTV-ErbB2 mice
- Preliminary evidence that TTR-IGF-IxErbB2 mammary tumors may grow faster than -ErbB2 tumors
- Evidence that TTR-IGF-IxErbB2 mammary tumors have increased levels of IGF-I compared to ErbB2 tumors

D) REPORTABLE OUTCOMES

None as yet

E) CONCLUSION

This study raises important questions regarding the epidemiological evidence suggesting that elevated circulating IGF-I is associated with increased breast cancer risk in humans, and experimental evidence showing that twice daily injection of IGF-I promotes mammary tumorigenesis and metastasis. Our data indicates that elevated circulating IGF-I doesn't alter ErbB2-induced tumorigenesis but it may alter established tumor growth rate. Unfortunately other studies that have studied circulating IGF-I and breast cancer risk in animal models have injected IGF-I and used different methods of mammary tumorigenesis including carcinogen (DMBA) or other oncogenes (SV40T antigen). Unfortunately, we can't directly compare our results with published studies due to the different methods of tumorigenesis, however, the conclusion is either that ErbB2-induced tumorigenesis is insensitivity to circulating IGF-I, or other studies using injected IGF-I were confounded by the transient spike in IGF-I caused by the non-physiologic method of administration. Further studies using additional mouse models are required to definitively address a role for circulating IGF-I in mammary tumorigenesis, however our data suggest that circulating IGF-I may not directly regulate risk, but rather may be an indicator of another risk factor. Further studies are required to test this hypothesis.

We are still very interested in discovering whether circulating IGF-I alters stem cell populations given a recent study in Nature showing that IGFs are dominant regulators of the human embryonic stem cell niche[4] and a previous Science article showing that elevated circulating IGF-II alters intestinal maturation and tumorigenesis by increasing the stem/progenitor cell population[2]. We will perform the mammary gland transplantation studies to assess mammary stem cell number during the next year using a no cost extension and when the TTR-IGF-I mice have been adequately backcrossed to the FVB/N genetic background.

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